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HSV Growth, Preparation, and Assay

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1. Introduction

Whether herpes simplex virus (HSV) is viewed as a pathogen or as a model eukaryotic system, it is virtually certain that any experimental work will require the virus to be grown and assayed. The following chapter is therefore seen as the fundamental first step before embarking on more intellectually and technically challenging technology. Its importance should not however be underestimated. It never fails to surprize us that people who describe themselves as virologists have little understanding of the basic requirements needed to attain a contamination-free, high-titer, low particle:plaque-forming units (PFU) ratio, genetically pure virus stock

HSV grows well in a wide variety of cell types to yield high-titer stocks. In general, HSV type 1 (HSV-1) grows to a higher titer than type 2 (HSV-2) and is less cell-associated, i.e., more infectious virus is released into the growth medium. Cell lines routinely used to grow HSV include BHK (hamster kidney), RK13 (rabbit kidney), Vero (monkey kidney), and CV1 (monkey kidney).

When HSV infects a single cell, the surrounding cells will also become infected by spread of progeny virus from cell to cell. This focus of infection normally causes cell necrosis, resulting in a hole in the monolayer with rounded cells at the periphery. Alternatively, certain virus strains can pass from cell to cell and cause fusion of the infected cells, resulting in a syncitium. For either type, these foci of infection are called plaques and are a measure of the number of infectious particles within a virus stock. The titer of a virus stock is expressed as the number of PFU per milliliter of virus (PFU/mL).

Spontaneous genomic mutations (point mutations, deletions, insertions) occur relatively frequently within a virus stock and, if nonlethal, they will be maintained. Therefore, to achieve genomic homogeneity, it is essential that a

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virus stock originates from a single virus plaque (single infectious particle) and that subsequent passage numbers are kept to a minimum. To ensure the purity of the isolate from which the stock will be derived, it must be stringently plaque-purified. This is done by serial dilution of the virus until preferably only one plaque is present on a monolayer. This plaque is picked, the virus titrated again, and a single plaque picked. A minimum of three rounds of stringent purification is usually required to yield a pure stock. Once a virus stock has been grown up from this plaque-purified isolate, it should be retained as an elite master stock and used as the only source of virus for generating working virus stocks.

The quality of virus stocks can also be adversely affected if the correct procedures are not followed when growing the virus Defective particles are generated when incomplete virus genomes are packaged If the DNA in the defective particle contains an origin of replication, it can be replicated in the presence of the standard virus, which supplies essential helper virus functions. All virus stocks should be grown from low multiplicity of infection (MOI) inocula. This optimizes amplification and packaging of complete virus genomes as opposed to defectives, during the several cycles of genomic replication required to generate a stock. The proportion of defective particles within a stock is a good indication of the quality of the virus. It is desirable for most experimental procedures to use stock with as low a particle:PFU ratio as possible. Wild-type stocks of HSV-1 with a ratio of 5:1 or less can be achieved, and a stock with a ratio >10:1 should be considered poor. For HSV-2, the average ratio of a good stock is <100·1

2. Materials

2.1. Reagents

1 ETC_{10}· Glasgow modified Eagle's medium with the addition of 10% newborn calf serum, 100 U/mL penicillin, 100 U/mL streptomycin and 10% tryptose phosphate broth (TP)
2 ETMC 10% Glasgow modified Eagle's medium with the addition of 10% newborn calf serum, 100 U/mL penicillin, 100 U/mL streptomycin, 10% TP, and 1% methylcellulose Since the methylcellulose needs to be heated to solubilize, 10X concentrated Eagle's medium is used The requisite amount of low-viscosity carboxymethylcellulose, sodium salt is dissolved in water to give a final concentration in the medium of 1% After autoclaving, the methylcellulose solution is substituted for water when making up the media
3 Phosphate-buffered saline (PBS)/calf serum PBS with the addition of 5% newborn calf serum
4 Brain heart infusion (BHI) agar
5 Blood agar: BHI agar containing 10% horse blood.
6. Giemsa: Giemsa's stain (Gurr)
7 Virkon

2.2. Equipment

1 Trays for Petri dishes
2 Bijoux racks.
3 Cell monolayer scrapers
4 Vortex.
5 Sonibath.
6 Stereo zoom plate microscope.
7 Centrifuge (2000 rpm), e g , Beckman GPR centrifuge
8 Centrifuge (12,000 rpm), e.g , Sorvall RC5C
9 CO_2 incubators
10 Roller bottle incubators
11. Class II hood
12 –70°C Freezer
13. Availability of an electron microscope (for particle counts)

3. Methods

Many tissue-culture lines can be used for the growth of HSV, but for the purpose of this chapter we will concentrate on BHK 21/C13 cells, which are routinely used in Glasgow and which give high yields of infectious virus. BHK 21/C13 cells are grown in ETC_{10} at 37°C in an atmosphere containing 5% CO_2.

For the preparation of large stocks of virus, 10 roller bottles of BHK cells (approx 3×10^8 cells/bottle) are used, which should yield 5–10 mL of stock at approx 10^9–10^{10} PFU/mL. Virus production on this scale requires an incubator capable of accomodating roller bottles If a suitable incubator is not available, it will be necessary to scale down the method appropriately.

Wild-type HSV-1 will grow over a large range of temperatures, between 31 and 39°C with little discernable effect on infectious virus yield. However, it is preferable to grow virus stocks at 31°C, since fewer defective particles are generated than at 37°C If roller bottle space at both 37°C (for growth of cells prior to virus inoculation) and 31°C (for virus growth) is not available, the virus can usually be grown at 37°C with only a marginal impairment in quality.

3.1. Growth of HSV Stocks

Good microbiological practice and sterile techniques need to be used throughout the procedure.

1. Seed each of 10 roller bottles with 3×10^7 BHK cells in 100 mL of ETC_{10} medium and add 5% CO_2 either from a central CO_2 line or from a cylinder In practice, this is done by attaching a sterile Pasteur pipet to the line, inserting the pipet into the bottle, and counting to 5[1]
2. Grow the cells at 37°C for 3 d until they form almost confluent monolayers.
3. Pour off the growth medium, and infect with virus at an MOI of 1 in 300 Assum-

ing 3×10^8 cells/bottle, add 10^6 PFU in 20 mL of fresh ETC_{10}. There is no need to add more CO_2

4. Incubate the infected cells at 31°C Cytopathic effect (CPE) should be apparent after 1–2 d, and the virus will be ready to harvest in 3–5 d when the cells have rounded up and are starting to detach from the plastic
5. The roller bottles should be shaken (unopened) until all the cells are in the medium If this proves difficult, sterile glass beads (approx 2-mm diameter) may be added and swirled around to detach the adherent cells
6 The medium containing the detatched cells should be poured into a sterile 200-mL centrifuge bottle (the glass beads if used will remain in the roller bottle) and spun at 2000 rpm for 10 min to pellet the cells Both the cell pellet and the supernatant should be kept
7 The supernatant should be poured into a sterile 250-mL centrifuge bottle and spun at 12,000 rpm, e g , in a Sorvall GSA rotor for 2 h The resultant pellet will consist of cell-released/supernatant virus (SV) and should be resuspended in 1 mL ETC_{10}/roller bottle
8. To harvest the cell-associated (CA) virus, the cell pellet from step 6, should be resuspended in a small volume (2–5 mL) of ETC_{10} This should be transferred to a suitable container (glass universal bottle) and sonicated thoroughly in a sonibath to disrupt the cells The sonicate should be spun at 2000 rpm for 10 min and the supernatant kept as fraction (1) of the CA virus To re-extract, a further 2–5 mL of fresh ETC_{10} should be added to the pellet, the solution sonicated, and the cell debris spun out again at 2000 rpm for 10 min This CA fraction 2 should be added to fraction 1
9 The CA and SV virus preparations may be kept separate or combined If they are to be kept separate, the virus pellet from step 7 should be resuspended in 5–10 mL of fresh ETC_{10} and sonicated briefly in a sonibath to disrupt the pellet If they are to be combined, then the pellet from step 7 can be resuspended directly by sonication in the CA fraction, since the overall resultant volume will be smaller Usually for HSV-1, SV and CA titers are similar For HSV-2, the CA titer is usually 10 times higher than SV

3.2. Sterility Checks

1. Sterility checks should be carried out on a new virus stock to ensure that it is free from bacterial or fungal contamination before storing at –70°C. This is done by streaking an inoculum of the virus on a blood agar plate using a sterile platinum loop and incubating the plate at 37°C for several days To test for fungal infections, the virus stock can be similarly streaked on a BHI agar plate and the plate incubated at room temperature for up to a week If the stock is contaminated with either bacteria and/or fungi, obvious colonies and/or hyphae will be seen on the plates Usually, a distinct smell will be obvious!
2. It is usual for contaminated stocks to be discarded, but if the virus is "irreplaceable," it can be filter-sterilized to remove bacterial or fungal contamination Unfortunately, this results in a large drop in titer and loss of volume, so it is only

worthwhile if the virus is very important. Clearance of contamination is achieved by passing the virus through a 0.2-μ pore size filter. It may be easier if the stock is first passed through a 0.4-μ filter.

Note: It is important always to wear safety goggles when carrying out this procedure, since there is a risk of the syringe detatching from the filter and spraying virus into the face

3. Mycoplasma contamination of virus stocks is hard to detect, although mycoplasma usually cause blood agar plates to discolor. If the cells used to grow virus test positive for mycoplasma, the virus stock and the cells should be immediately discarded. If the virus is "irreplaceable," it is possible to extract viral DNA, which can be used to transfect clean cells to obtain a mycoplasma-free, virus stock

3.3. Viability

To reduce the number of freeze-thaw cycles, virus stocks should be aliquoted maximally into 1-mL amounts and stored at –70°C.

Note: HSV should never be stored at –20°C, since infectivity will be lost very rapidly. Aliquoted vials should be frozen quickly, and when being thawed, they should be warmed rapidly and kept at 0–4°C until use. The amount of time the virus is at 0–4°C should be kept to a minimum, but it can remain at 4°C for 24 h without a significant drop in titer.

3.4. Titration of Virus Stocks

To quantitate the amount of infectious virus within a stock, it is necessary to titrate the stock on cell monolayers, and count the number of plaques on plates that have been fixed and stained to make the plaques easily visible under a microscope. The titer is expressed as PFU/mL of virus.

1. Seed 60-mm plastic Petri dishes with 3×10^6 BHK cells in 5 mL of ETC_{10}
2. Incubate the plates overnight in a 37°C incubator in an atmosphere with 5% CO_2 The cells should form just subconfluent monolayers.
3. Serial dilutions of virus are made in PBS/calf serum, which is aliquoted in 0.9-mL amounts into the calculated number of bijoux bottles
4. Dilute the virus (1/10) by adding 100 μL of virus to a 0.9-mL aliquot of PBS/calf serum (giving a 10^{-1} dilution). Recap the bottle, and vortex to mix. Using a fresh tip, take 100 μL of the 10^{-1} stock and transfer into another 0.9-mL aliquot of PBS/calf serum giving a 10^{-2} dilution Vortex, and so on Continue with this serial dilution procedure until the appropriate range of dilutions has been achieved. For a large-scale virus preparation, which may yield up to 10^{10} PFU/mL, it is necessary to titrate out to a dilution of 10^{-7} or 10^{-8}

   Note: The tip should be touched against the side of the bottle and not into the liquid, since droplets on the outside of the tip can be carried over, introducing inaccuracies.
5. Pour the growth medium off the 60-mm plates.

6. Plate out 100 µL of the serially diluted virus stock onto the BHK monolayers, taking care not to dislodge the cells from the plates when delivering the inoculum through an Eppendorf tip Starting with the highest dilution and working back to the most concentrated, it is not necessary to change tips, since any carryover will be insignificant. Rock the trays of plates back and forth gently to ensure even coverage of virus
7. Put into a 37°C incubator for 1 h to allow absorption of the virus onto the monolayers
8. Add 5 mL of ETMC 10% to each plate The methylcellulose stops progeny virus from the plaques formed from the inoculum from spreading through the medium and resulting in trailing plaques or secondary satellite plaques
9. Place the titration plates in a CO_2 incubator at the appropriate temperature Wild-type virus can be titrated at 31 or 37°C Temperature sensitive virus is usually titrated at the permissive (e g , 31°C) and nonpermissive (e g , 38.5°C) temperature Incubate plates for 2 d at 37°C or 38 5°C, and 3 d at 31°C
10. The viscosity of the methylcellulose makes it difficult for stain to permeate through to the cell monolayers, and it is therefore preferable to pour off the overlay medium prior to the addition of 2–3 mL of Giemsa's stain The decanted medium will contain virus, and should be autoclaved or treated with an appropriate viricidal agent (e g , Virkon)

    The stain should be left on the plates for 2–24 h at room temperature Staining fixes the cells, and any virus remaining on the plates will be inactivated The stain can be washed off directly under running tap water.
11. Using a plate microscope, count the plaques on the monolayers by inverting the dish, and with a water-soluble pen, mark off each plaque as it is counted. It is best to count the dilutions with 20–200 plaques/plate, since too many or too few plaques give less accurate counts Ideally, duplicates of each dilution should be counted and the average count used In practice, it is usually sufficient to count the number of plaques from two plates with serial dilutions, e.g , 10^{-5} and 10^{-6}. The accuracy of the titration can be measured in this way

    Note: Plaques should always be counted using a microscope Although some may be visible to the naked eye, the size of plaques can vary considerably, and many will be missed if a microscope is not used
12. The titer should be calculated as follows

$$20 \text{ plaques on the } 10^{-7} \text{ plate and } 200 \text{ on the } 10^{-6} \text{ plate} = 2 \times 10^{8} \text{ PFU in the } 100\ \mu\text{L inoculum} \quad (1)$$

    The titer is therefore 2×10^{9} PFU/mL

3.5. Particle Counts

Virus suspensions are mixed with equal volumes of a 1% solution of sodium silicotungstate and a suspension of latex beads of known concentration We use a solution of 1.43×10^{11} particles/mL. A droplet of this suspension is placed on an electron microscope grid and, after 5 min (when the particles have

settled), the excess suspension is removed and the particles are counted. The latex beads are of course used as the reference count

A wild-type stock of HSV-1 should ideally have a particle:PFU ratio of <10:1, and for HSV-2 this figure should be <100.

3.6. Single and Multicycle Growth Experiments

To assay the in vitro growth phenotype of a particular virus stock, it may be necessary to determine its growth kinetics over one or more replication cycles compared with a known standard. This is achieved by infecting multiple plates of cells with virus, under the same conditions, but harvesting at different time-points postinfection. The progeny virus from the different time-points is titrated to monitor progression of the infection.

A single-cycle growth experiment involves infecting every cell in a monolayer and monitoring the growth during one round of replication. To do this, cells are inoculated with an MOI of 5 or 10 PFU/cell to ensure that every cell is infected and the progress of the infection is normally monitored during 24 h.

A multicycle growth experiment amplifies the effect of any small impairment during several rounds of replication. In this case, cells are infected at a low MOI (usually 0.01–0 1 PFU/cell), and the infection is monitored over 72 h.

The method for both is the same with only the virus inoculum and the points of harvest varying.

1. Count a BHK 21/C13 cell suspension and seed 35-mm plates with 2×10^6 cells/dish in 2 mL of ETC_{10} Seed a single plate per time-point for each virus being assayed Especially for large experiments where several viruses are being compared, it is advisable to label the plates at this stage, since it saves time when inoculating with virus
2. Incubate overnight at 37°C
3. Pour off the growth medium
4. Inoculate with virus, e.g , 2×10^6 cells infected at a MOI of 5 PFU/cell means an inoculum of 1×10^7 PFU/plate. Therefore, it is necessary to dilute the virus to 1×10^8 PFU/mL and add 100 µL/plate Make sufficient diluted virus for all of the time-points, so that the inocula going onto a series of plates is fom a single virus solution
5. Incubate at 37°C for 1 h to allow the virus to absorb.
6. Wash the plates with 2 mL of PBS/calf serum to remove any unabsorbed virus
7. Overlay the plates with 2 mL of ETC_{10} (accuracy here is very important) This is 0 h on the time scale
8. Incubate at the appropriate temperature (normally 37°C)
9. Harvest the virus samples at the designated time-points by scraping the cell monolayer into the medium and transferring the suspension to a clearly labeled sterile bottle that is suitable for sonication (black cap vial)

   Time-points for harvesting are arbitrary, but for a high MOI (single-cycle)

experiment, 0-, 2-, 4-, 6-, 12-, and 24-h points are usual, and in some cases, 8- and 16-h points may also be required For a low-MOI (multicycle) experiment, 0-, 4-, 8-, 12-, 24-, 48-, and 72-h samples are usual.

10 Sonicate the samples thoroughly in a sonibath to disrupt the cells, and release the virus into the medium. Store the samples at –70°C until they can be titrated.

11 Titrate the virus as described above, and calculate the titers at each time-point Since virus from 2×10^6 cells is harvested into 2 mL, the final titer per milliliter is equivalent to its titer per 10^6 cells

12 Plot out the titers on a log graph scale with PFU/10^6 cells ($\log_{10}$) on the *y*-axis and time (in hours) on the *x*-axis

References

It will be obvious that we have not included any references Since the step-by-step procedures explained in this chapter are fundamental and have been in operation for many years, we are assuming that it will be unnecessary for the reader to require more detailed information. Most of the basic references are in papers published over 20 years ago!

2

HSV Entry and Spread

Christine A. MacLean

1. Introduction

This chapter deals with assays commonly used to follow herpes simplex virus type 1 (HSV-1) entry into and spread between cells in tissue culture These are complex processes, known to involve several of the 20 or more HSV-encoded membrane proteins (*see* refs. *1* and *2* for recent reviews). HSV entry is mediated by a number of proteins on the surface of the virus particle. Recognition of and binding to target cells are known to involve at least three glycoproteins—gB, gC, and gD. gC mediates the initial interaction with cells, recognizing heparan sulfate proteoglycans on the cell surface. gB also interacts with heparan sulfate proteoglycans, and can substitute for gC in gC negative viruses. This initial, heparin-sensitive attachment to cells is relatively weak, and is followed by a more stable attachment to cells, apparently mediated by gD. Following attachment, the virus particle fuses with the cell membrane to mediate entry. Fusion is known to require gB and gH/gL, and possibly also gD, but their precise functions are uncertain. The roles of other virus-encoded membrane proteins in entry are unclear, but it is possible that different proteins may be required for entry into different cell types.

Following infection, spread of progeny virus in tissue culture occurs via both the release of mature infectious virus particles into the extracellular medium and the direct cell-to-cell spread of virus UL20 plays a role in membrane trafficking events involved in the maturation and egress of virus particles, whereas several virus membrane proteins are probably involved in the membrane fusion event required for cell-to-cell spread, including gB, gD, gE/gI, gH/gL, and gK.

This chapter will describe assays that address virus entry, in terms of the initial attachment of virus to cells (adsorption) and the subsequent fusion between

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the virus and cell membranes (penetration), and virus spread, in terms of both intracellular and extracellular virus yields (virus release) and virus growth under conditions that limit extracellular spread of virus (cell-to-cell spread). Detailed methodology is provided for the assays used in our laboratory, although some attempt will be made to refer to procedures used by others. The assays described here involve the use of tissue-culture cells, the growth of virus stocks, and extensive virus titration. The reader should therefore be familiar with the procedures described in Chapter 1.

2. Materials

1 Cells We standardly use baby hamster kidney 21 clone 13 (BHK C13) cells, although any cell line permissive for HSV infection should be suitable BHK C13 cells are grown in ETC_{10} *(see* step 2*)*, at 37°C in a humidified atmosphere containing 5% (v/v) carbon dioxide Cell monolayers are seeded at 1×10^6 cells/35-mm Petri dish or a well of a six-well tray, or 2×10^6 cells/60-mm Petri dish, 20–24 h before use Cell monolayers are used when about 80–90% confluent, and we assume approx 2×10^6 cells/35-mm dish

2 Media ETC_{10} Eagle's medium supplemented with 10% newborn calf serum, 5% tryptose phosphate broth, 100 U/mL penicillin, and 100 mg/mL streptomycin

EC_5/EC_2 Eagle's medium supplemented with antibiotics, and either 5 or 2% newborn calf serum, respectively

Emet/$5C_2$ Eagle's medium containing one-fifth the normal concentration of methionine and 2% newborn calf serum

MC_5/MC_2 Eagle's medium supplemented with antibiotics, 1 5% carboxymethylcellulose, and either 5 or 2% newborn calf serum, respectively

EHu. Eagle's medium supplemented with antibiotics and 10% pooled human serum

3 PBS 170 m*M* NaCl, 3 4 m*M* KCl, 10 m*M* Na_2HPO_4, 1 8 m*M* KH_2PO_4, supplemented with 6 8 m*M* $CaCl_2$ and 4.9 m*M* $MgCl_2$

4 Citrate buffer. 40 m*M* citric acid, 135 m*M* NaCl, 10 m*M* KCl, pH 3 0

3. Methods

The methods described here are based on the use of HSV-1 strain $17syn^+$ in BHK C13 cells. It is important to remember that growth characteristics and/or kinetics of entry may differ when using different strains of HSV-1 or different cell types

Before undertaking these procedures, consult local regulations for the safe handling of HSV. We generally work with the virus on the bench or in a class II biological safety cabinet, and inactivate all waste either by steeping overnight in a 1% solution of vircon or chloros, or by autoclaving. The most obvious risks from HSV are from splashes to the eye, or contact with areas of broken skin (e.g., cuts, eczema). Because of the large numbers of infected monolayers

that may need to be handled at once in some of the procedures below, it is rather easy to be a little careless.

Unless otherwise stated, manipulations are carried out at room temperature, as rapidly as possible. Seed stocks of virus or cells are generally handled in a biological safety cabinet using sterile technique, but all other procedures are conducted on the bench using good microbiological practice.

3.1. Adsorption of Radiolabeled Virions to Cells

This assay measures the proportion of total virus particles that bind to cells with time. Purified radiolabeled virus particles are allowed to bind to cells for given periods of time, the cells washed extensively to remove unbound virus, and the cells then lysed and the amount of bound radiolabel measured. The radiolabeled virions used in these experiments should be free from contaminating cell membranes or debris. We generally use ^{35}S-methionine-labeled virions, purified by Ficoll gradient centrifugation *(3),* but other radiolabels (e g., ^{3}H-thymidine) and/or different gradient purification procedures are also suitable (e.g., *see* refs. *4–6*). We generally find that around 15–20% of radiolabeled virions bind to cells within 60–90 min at 37°C.

3 1.1. Step 1: Preparation of Radiolabeled Virions

1. Infect 80–90% confluent cell monolayers in 80-oz roller bottles (assume around 2×10^8 BHK C13 cells/roller bottle) at 0 001 PFU/cell in EC_2 at 31°C (*see* Note 1).
2. Once plaques become visible (12–24 h pi), remove the medium and wash the cells twice with, and subsequently maintain them in, Emet/$5C_2$. Approximately 2–4 h later, add ^{35}S-methionine (Amersham, SA >1000 Ci/mmol) to a final concentration 10–20 mCi/mL. A total volume of 20 mL is usually sufficient, but ensure that the cells do not dry out.
3. Once all the cells appear rounded, but still attached to the roller bottles (3–4 d pi), carefully remove the culture supernatant (avoid detaching the cells), and pellet the cell debris by centrifuging the supernatant in a Fisons coolspin centrifuge (or equivalent) at 2000 rpm for 30 min at 4°C. Care should be taken to avoid cell debris in virus stocks membrane fragments can copurify with virions on Ficoll gradients, and excessive cell debris can trap/sequester virus in large aggregates, resulting in low yields of purified virions
4. Again, carefully remove the supernatant, and then pellet the virus by centrifugation at 12,000 rpm for 2 h at 4°C in a Sorvall GSA rotor. Remove all the supernatant, add 1 mL of Eagle's medium without phenol red, and then very gently scrape the virus pellet into the medium and allow the virus to resuspend overnight at 4°C Virions should be handled very gently at all stages to avoid damage to the virus envelopes.
5. Prepare 35-mL continuous 5–15% Ficoll gradients (Ficoll 400, in Eagle's medium without phenol red) in transparent centrifuge tubes that can be easily pierced by a syringe needle, and cool on ice or at 4°C. We generally use Beckman Ultra-

clear™ centrifuge tubes Gently pipet the virion suspension until homogeneous, layer it onto the Ficoll gradients, and centrifuge for 2 h at 12,000 rpm, in a Sorvall AH629 rotor at 4°C The number of roller bottles of virus loaded per gradient will depend on the yield of virus expected For 17syn$^+$ wild-type, virus from 2–5 roller bottles would normally be loaded onto a single 35-mL Ficoll gradient (approx 1–5 × 10^9 PFU at the end of step 4)

6 Visualize the virion band under a light beam (*see* ref *3* and Note 2) Carefully remove the virion band by side puncture, using a 5-mL syringe and broad (18/19G) gage needle, dilute the virus in Eagle's medium without phenol red, and then recover the virus by centrifugation at 21,000 rpm for 16 h in a Sorvall AH629 rotor at 4°C.

7 The virus pellet should appear as an opaque halo at the base of the centrifuge tube Remove the supernatant carefully, and dry the tube with a tissue to remove excess liquid (avoid disrupting the pellet) Add 500 mL ETC_{10}, gently scrape the virus into the medium, and allow it to resuspend overnight at 4°C

8 Very gently, resuspend the virus preparation until homogeneous, using an Eppendorf pipet, and then determine
   a. The quality of the preparation, by electron microscopy,
   b Particle numbers (particles/mL), by electron microscopy,
   c The virus titer (PFU/mL), and
   d Radioactivity (counts/min/mL), by liquid scintillation counting

9 Virions can be stored at –70°C until use

3.1.2. Step 2: Adsorption of Radiolabeled Virions to Cells

1 Remove the medium from 90–100% confluent monolayers in six-well trays (*see* Note 3), and add ETC_{10} containing 1% BSA for 30–60 min at 37°C This step is included to reduce nonspecific binding of virions, although in practice, we find little difference if this step is omitted

2. Dilute the radiolabeled virions in prewarmed ETC_{10}. The amount of virus added will vary in terms of counts. When comparing different viruses, we aim to use comparable particle numbers, while trying to keep the counts within the range 10–1000 cpm/μL (20–200,000 cpm/well). This is usually 10^1–10^3 particles/cell, and does not reach saturation binding (*see* refs *7* and *8*)

3. Remove the blocking medium Since volume influences adsorption rates, all wells should be drained thoroughly

4 Add 200 μL prewarmed virus/well, in triplicate for each time-point, plating different time-points on separate trays (since shaking will affect the rate of adsorption) Plate all viruses for each time-point together. the first set added should be the last time-point harvested, whereas the last set added should be the first time-point harvested (*see* Note 4) Transfer monolayers to 37°C; this represents 0 time

5 At the relevant time-points, remove the virus supernatant using an Eppendorf pipet, and transfer to a scintillation vial

6 Wash the cells three times with 1 mL PBS, shaking the trays for 5–10 s each time, and transfer each wash to a scintillation vial

7 Harvest the cells (and bound virus) by scraping into 300 µL PBS/1% (v/v) SDS, and transfer this to a scintillation vial.
8 Add 4 mL Ecoscint™-A (National Diagnostics, Atlanta, GA) to each vial, vortex briefly, and count each sample in a liquid scintillation counter for 1 min
9. The percentage of bound virus at each time-point is calculated from:

$$(\text{cpm bound}/\text{total recoverable counts}) \times 100 \quad (1)$$

where cpm bound = cpm in cell harvest and total recoverable counts = (cpm in virus supernatant + cpm in washes1/2/3 + cpm bound).

3.2. Adsorption of Infectious Virus to Cells

In this assay, virus is allowed to attach to cells for given periods of time, the cells washed extensively to remove unbound virus, and the amount of bound virus then measured in terms of subsequent plaque formation. Either crude virus preparations or gradient purified virions can be used as input virus.

1. Remove the medium from 90–100% confluent monolayers in six-well trays, and drain all wells thoroughly
2 Briefly sonicate virus stocks before use (30–60 s), and dilute the virus in prewarmed ETC_{10} to 150–200 PFU/200 µL (*see* Notes 5 and 6).
3. Add 200 µL virus/well, in triplicate for each time-point, plating different time-points on separate trays Plate all viruses for each time-point together the first set added should represent the last time-point handled, whereas the last set added should be the first time-point harvested. Transfer monolayers to 37°C, this represents 0 time.
4 At relevant time-points, remove the virus using an Eppendorf pipet, and discard
5. Wash the cells three times with 2 mL PBS, shaking the trays for 5–10 s each time
6 Drain all wells thoroughly Overlay the monolayers with 2 5 mL MC5 (or MC_2 if the cells are very confluent), and incubate at 37°C until plaques are clearly visible (usually 2 d pi).
7 Stain the cells by adding 1–2 mL Giemsa stain, leaving the cells at room temperature for 2–24 h before washing.
8 Count the plaques under an inverted microscope.
9. The percentage of infectious virus binding to cells at a given time is calculated from

$$(\text{avg. no. of PFU at given time}/\text{avg. no. of PFU at peak or final time-point}) \times 100 \quad (2)$$

3.3. Modifications of the Adsorption Assays

Sections 3.1. and 3.2. describe adsorption of virus at 37°C, under standard conditions. It is obviously possible to modify these procedures in a number of ways: e.g., to wash in the presence of reagents that may interfere with binding (e.g., heparin) or to slow adsorption by incubating at 4°C (*see* refs. *9* and *10*).

To carry out these assays at 4°C, both cells and virus should be precooled before addition of virus to cells, and the experiments carried out in a

4°C cold room. We do not cool the cells on ice, as described by others, since we find that our BHK C13 cells do not survive such treatment well. Time-points are washed at 4°C, before transferring to room temperature for harvesting (Section 3.1.) or addition of prewarmed MC5 (Section 3 2.). A reasonable time course would be 0, 15, 30, 45, 60, 90, 120, 180, and 240 min after virus addition. We find BHK C13 cell monolayers do not survive longer periods at 4°C

3.4. Penetration

Virus penetration is measured as the rate at which attached virus becomes resistant to inactivation by low pH *(11,12)*. Virus is bound to cells at 4°C, a temperature at which very little penetration should occur. Cells are then shifted to 37°C, to allow penetration to begin, and at various times the monolayers are treated with low-pH buffer to inactivate virus that has not penetrated the cell Virus penetration is measured in terms of subsequent plaque formation and expressed as a percentage of the number of plaques formed on control monolayers

To minimize penetration during the 4°C adsorption stage, steps 1–4 below are carried out in a 4°C cold room Warm clothing and gloves are strongly advised!

1. Remove the medium from 90–100% confluent cell monolayers in six-well trays, replace with cold (4°C) medium and incubate at 4°C for 15–30 min
2. Briefly sonicate virus stocks (30–60 s), and dilute in precooled ETC_{10}, to 150–200 PFU/200 µL (*see* Note 7).
3. Remove the medium from the wells, and drain thoroughly Add 200 µL virus/well, and incubate at 4°C for 60 min (*see* Note 8) Note that for each virus, one six-well tray will represent one time-point
4. Remove the virus, and wash the cells twice with cold PBS
5. To start penetration, add 2 mL prewarmed ETC_{10} (37°C), and transfer the cells to a 37°C incubator This represents 0 time Again, add the overlay to all viruses for each time-point together the first set added should represent the last time-point handled, whereas the last set added should be the first time-point harvested (*see* Note 9)
6. At the relevant time-point, remove the medium from the trays, and add 1 mL PBS to three wells (control), and 1 mL citrate buffer, pH 3 0, to the remaining 3 wells of each tray Incubate for 3 min at room temperature with gentle shaking It is important to include a set of PBS control wells for each time-point, since absolute plaque numbers do vary from tray to tray (*see* ref *13*)
7. Remove the buffer and wash the cells twice with PBS, shaking 5–10 s each wash
8. Drain the wells thoroughly, then add 2 5 mL MC5, and incubate at 37°C until plaques are clearly visible
9. Stain and count as described above (Section 3.2)
10. Penetration is measured as the percentage of acid-resistant virus with time

$$\text{Each time-point} = (\text{avg PFU on low pH-treated wells} / \text{avg PFU on PBS-treated wells}) \times 100 \quad (3)$$

3.5. Virus Release

To measure the efficiency of release of virus particles during infection, the percentage of total infectious progeny virus that is present within the extracellular medium is measured with time, following infection at either high or low multiplicity. Infection at high multiplicity (5–20 PFU/cell) follows infection through one infectious cycle. Infection at low multiplicity (0.001 PFU/cell) allows multiple cycles and may amplify small differences in overall virus growth

1. Briefly sonicate virus stocks (30–60 s), and dilute to either 5×10^7 PFU/mL (for infection at 5 PFU/cell, assuming 5×10^6 cells/60-mm Petri dish), or 1×10^4 PFU/mL (for infection at 0.001 PFU/cell) (*see* Notes 10 and 11)
2. Remove the medium from 90–100% confluent cell monolayers in 60-mm Petri dishes. Add 0.5 mL virus/plate, and incubate at 37°C for 1 h.
3. Remove the virus inoculum. Add 1 mL citrate buffer, pH 3.0, per plate, and incubate at room temperature for 2–3 min (*see* Note 12).
4. Remove the buffer, wash the cells twice with PBS, and drain the monolayers thoroughly.
5. Add 2 mL EC_5/plate (EC_2 if the monolayers are very confluent), and incubate the cells at 37°C.
6. At the relevant time-points, remove the medium to a glass bijou bottle (or alternative vial suitable for sonication), and measure the volume. Store on ice. This represents the released or supernatant, virus (SV).
7. Wash the monolayer gently twice with PBS—if any cells detach, recover these by centrifugation (e.g., Fisons coolspin, 2000 rpm, 5–10 min). Add 2 mL EC_5 to the monolayer, scrape the cells into the medium (e.g., using a commercial cell scraper, or a plunger from a sterile syringe), and transfer to a glass bijou. Pool any cells recovered from the washes. Store on ice. This represents the cell-associated (CA) virus.
8. Sonicate the SV stocks briefly (30–60 s), and the CA stocks until clarified (around 3×60 s). Store the SV and CA virus stocks at –70°C until they can be titrated.
9. Quantitate the yields of infectious virus by titration. This is basically the method described in Chapter 1 with some minor modifications. Prepare serial 10-fold dilutions of virus in a total volume of 1 mL ETC_{10} in 5-mL bijou bottles, mixing the dilutions by swirling. Because of the large number of titrations being handled, it is relatively easy to contaminate your hands with liquid from the lids when opening and closing the vials—swirling is less likely than vortexing to result in virus contamination on the lids of the vials. Virus should then be plated in duplicate on 60-mm Petri dishes, using 200 µL/plate and adsorbed for 1 h at 37°C. To minimize secondary spread of virus, the inoculum is removed before overlaying with MC_5. Note that MC_5 contains 1.5%, not 1%, carboxymethylcellulose.
10. Calculate the yield of virus in the CA and SV samples, since well as the percentage of progeny virus that is released at the different time-points.

$$\text{Virus yield} = \text{titer} \times \text{volume}$$
$$\%\text{SV} = [\text{SV yield/total virus yield (SV + CA yield)}] \times 100 \quad (4)$$

3.6. Cell-to-Cell Spread

Cell-to-cell spread can be assayed by infecting cells at low multiplicity and allowing the virus to grow under conditions that limit the extracellular spread of virus. We standardly use commercial pooled human serum, which contains high levels of neutralizing antibody to HSV in the overlay medium Neutralizing monoclonal antibodies (MAb) could also be used.

1 Briefly sonicate the virus stocks (30–60 s), and dilute to 1×10^4 PFU/mL (0 001 PFU/cell) (*see* Notes 13 and 14)
2. Remove the medium from 90–100% confluent cell monolayers in 60-mm Petri dishes Add 0 5 mL virus/plate, and incubate at 37°C for 1 h
3 Remove the virus inoculum, and add 1 mL citrate buffer, pH 3 0, per plate, and incubate at room temperature for 2–3 min
4 Remove the buffer, wash the cells twice with PBS, and drain the monolayers thoroughly
5 Add either 2 mL ETC_{10} or 2 mL Ehu/plate, and incubate the cells at 37°C
6 At the relevant time-points, remove the medium If desired, the supernatant from the ETC_{10}-treated plates can be kept, and treated as in Section 3 5 It is also a good idea to titrate one or two of the later EHu supernatants to monitor neutralisation
7 Wash the monolayers gently twice with PBS—if any cells detach, recover these by centrifugation (e.g., Fisons coolspin, 2000 rpm, 5–10 min) Add 2 mL EC_5 to the monolayer, scrape the cells into the medium, and transfer to a glass bijou Pool any cells recovered from the washes. Store on ice
8. Sonicate the SV stocks briefly (30–60 s), and the CA stocks until clarified (around 3–60 s) Store at –70°C
9 Quantitate the yields of infectious virus by titration (*see* Section 3 5 , step 9)

$$\text{Virus yield} = \text{titer} \times \text{volume} \quad (5)$$

Calculate the ratio of CA yields under human serum compared to normal medium, for each virus

4. Notes

1 If preferred, radiolabeled virions can be prepared following infection at high multiplicity (low multiplicity of infection is simply more economical with virus stocks) In this case, infection is carried out at 37°C Use 5 PFU/cell, infect in Emet/$5C_2$, add the radiolabel at 4 h pi, and harvest around 24 h pi
2. Large pellets after Ficoll gradient centrifugation can suggest either the presence of cell debris or overheating of the Ficoll during preparation of the solutions This can considerably reduce the yield of purified virions Ficoll solutions can be prepared by leaving overnight in the refrigerator to dissolve, and only minor warming with stirring is then required to get the Ficoll into solution Solutions are cooled to 4°C before gradients are prepared
3 Petri dishes (35-mm) can be used instead of six-well trays We use the latter, since they are easier to handle for the washing stages